AD_____

Award Number: W81XWH-13-1-0414

TITLE: Testing Brain Overgrowth and Synaptic Models of Autism Using NPC's and Neurons from Patient-Derived IPS Cells

PRINCIPAL INVESTIGATOR: Fred H. Gage, Ph.D.

CONTRACTING ORGANIZATION: The Salk Institute for Biological Studies
La Jolla, CA 92037

REPORT DATE: October 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2014	Annual	15 Sep 2013 - 14 Sep 2014

4. TITLE AND SUBTITLE
Testing Brain Overgrowth and Synaptic Models of Autism Using NPC's and Neurons from Patient- Derived IPS Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0414

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Fred H Gage

E-Mail: gage@salk.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Salk Institute for Biological Studies

La Jolla, CA 92037

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Autism and autism spectrum disorders (ASD) are complex neurodevelopmental diseases that affect about 1% of children in the United States. Such disorders are characterized by deficits in verbal communication, impaired social interaction, and limited and repetitive interests and behavior. Recent studies have led to two major hypotheses for autism pathogenesis: early brain overgrowth and synaptogenesis defects. The goal of this project is to produce human cellular models of non-syndromic ASD. We used cellular reprogramming to develop iPSCs from ASD patients (and non-autistic controls) for the production of patient-derived neural progenitors (NPCs) and neurons to study cellular phenotypes that directly test whether brain overgrowth and/or synaptogenesis mechanisms are found in ASD NPCs and neurons. Patient-derived NPCs and neurons from these ASD and control individuals will be used for the functional characterization of iPSCs-derived NPCs and neurons from ASD and control individuals for potential autism-specific defects in proliferation, neural development and synaptogenesis, and for gene expression studies. We have made signficiant progress on these aims in the first year.

15. SUBJECT TERMS
Autism, induced pluripotent stem cells, gene expression.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	9	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

1. **INTRODUCTION:**

Autism and autism spectrum disorders (ASD) are complex neurodevelopmental diseases that affect about 1% of children in the United States. Such disorders are characterized by deficits in verbal communication, impaired social interaction, as well as limited and repetitive interests and behavior. Recent studies have led to two major hypotheses for autism pathogenesis. First, early brain overgrowth appears to be a critical feature in the development of ASD. This hypothesis is based on MRI studies of autistic individuals (Courchesne et al. 2001; Schumann et al. 2010). A second model for the development of autism is synaptic dysregulation. This hypothesis is based on several lines of evidence, including the finding that mutations in genes coding for proteins that play important roles in synaptogenesis and synaptic function, such as MeCP2, Shank3, and the gene families for neurexin and neuroligin, are found in individuals with syndromic ASD. These pathogenic mechanisms are not mutually exclusive and may both be important for the development of ASD. The major impediment to testing these and other hypotheses about autism is the lack of relevant animal and cell models. The direct study of live brain tissue from ASD patients is impossible, and no suitable animal models can adequately reproduce the complicated structure and function of the human brain. Recently, reprogramming of human somatic cells to a pluripotent state by over-expression of specific genes into induced pluripotent stem cells, or iPSCs (Takahashi et al., 2007), has provided an exciting opportunity to produce a relevant human cellular model of complex human neurogenetic diseases (Marchetto et al. 2011). Now, iPSCs have been generated for several neurological disorders and diseases, including Rett syndrome, a syndromic ASD (Marchetto et al. 2010). The objective of this project is to produce human cellular models of ASD. Here we used cellular reprogramming to develop iPSCs from non-syndromic ASD patients (and non-autistic controls) for the production of patient-derived neural progenitors (NPCs) and neurons to study cellular phenotypes and test whether brain overgrowth and/or synaptogenesis deficits are found in ASD NPCs and neurons.

2. **KEYWORDS:**

Autism spectrum disorder, ASD, neurodevelopmental disease, disease modeling, induced pluripotent stem cell, iPS, human neurons, disease-in-a-dish, brain overgrowth.

3. **OVERALL PROJECT SUMMARY**

During the first year of the research grant we focused on the functional characterization of the iPSC-derived neuroprogenitors (NPCs) and neurons from ASD patients and age/gender matched neurotypical individuals. We have screened individuals for potential autism-specific defects in proliferation, neural development and synaptogenesis as proposed in the Statement of Work (SOW), Task 1.

Task 1: Functional characterization of iPSCs-derived NPCs and neurons from ASD and control individuals for potential autism-specific defects in proliferation, neural development and synaptogenesis (months 1-16).

1a. *Characterize ASD-iPSCs from 7 ASD patients and 6 controls (at least 2 clones for each individual) for pluripotency and neural differentiation potential (months 1-6).*

We recruited 8 ASD patients with quantitative MRI-validated early brain enlargement ranging from mild to macrencephaly and 5 age/gender-matched control individuals for skin biopsies and phenotypic characterization based on the stability of the karyotype. The ASD donors displayed larger brain size compared to the normal average brain size of typically developing control subjects at any given age (**Figure 1a, b Image courtesy from Eric Courchesne,** University of California Autism Center of Excellence**, La Jolla, CA**). Copy Number Variation analysis using DNA extracted from the donors' whole blood did not show the presence of any rare structural variant known to be associated with ASD unknown etiology (data not shown). ASD and non-affected control fibroblasts were transduced with 4 retroviral reprogramming vectors (Sox2, Oct4, c-Myc and Klf4), as described elsewhere [1]. Following 2 to 3 weeks of culture in human embryonic stem cells (hESC)-supporting conditions, compact refractile ESC-like colonies emerged from a background of fibroblasts. IPSC colonies were then manually picked and cultured under feeder-free conditions. Cells were mechanically expanded for at least 10 passages and tested for the expression of pluripotent markers. We obtained several clones that continuously expressed pluripotent markers, such as Nanog, Lin28, Tra-1-81 and Sox2 from each control wild-type (WT)-iPSCs and ASD-iPSCs. We excluded all karyotypically unstable clones from further experiments (**Figure 2**).

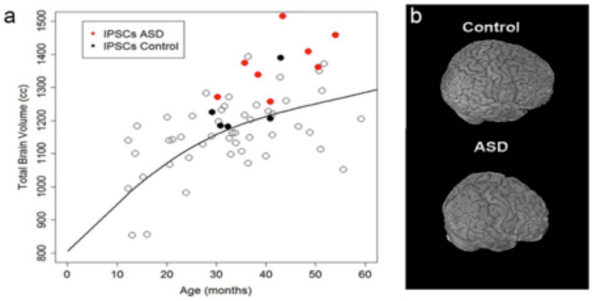

Figure 1. Derivation of NPCs from ASD and control subjects. (**a**) Left panel, scatterplot of Total Brain Volume (TBV) across ages. Open black circles indicate brain size of typically developing subjects. Black solid dots represent control donors. Red solid dots represent ASD donors. (**b**) Three-dimensional reconstructions of the brain from one control donor and one ASD donor.

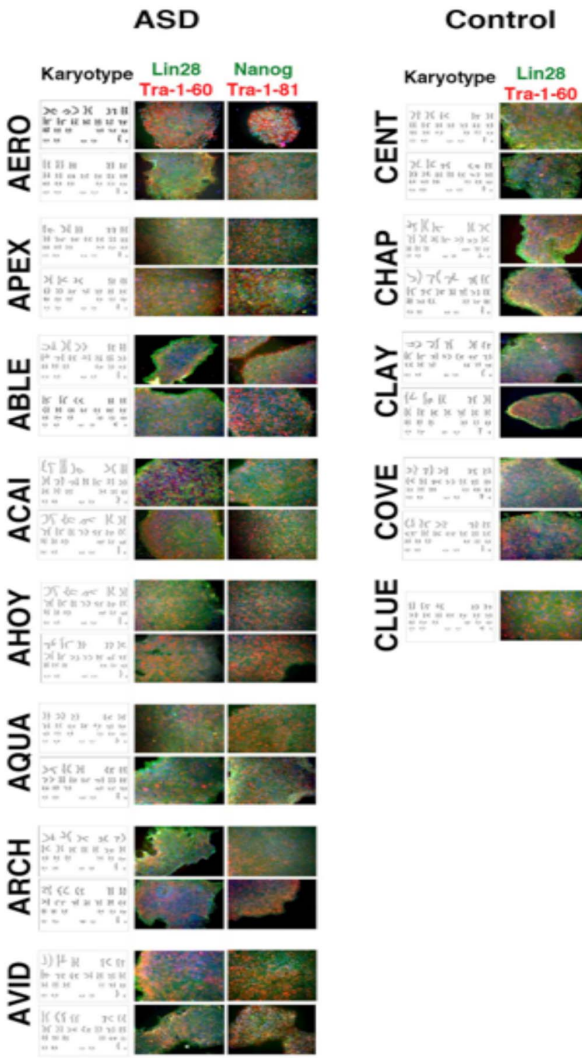

Figure 2. Characterization of iPSCs derived from controls and ASD subjects. (**a**) Quality control of fibroblast reprogramming showing normal karyotype and expression of pluripotent markers for at least 2 clones of each iPSC. Scale bar: 20 μm.

The data described in Task 1a was generated and analyzed in Dr. Gage laboratory, Salk Institute, La Jolla, CA.

1b. *Perform NPC differentiation using the iPSCs clones characterized in 1a and access cell proliferation using the methods described below (months 6-12).*

We used our previous published protocol to generate NPCs from iPSCs [2] in the presence of Noggin. Briefly, we initiated neural differentiation by plating 1-week-old embryoid bodies (EBs) treated with Noggin. After a week in culture, EB-derived rosettes became apparent in the dish. Rosettes were then manually collected, dissociated and re-plated. The NPCs derived from rosettes formed a homogeneous population after a few passages from patients and controls, and continued to proliferate in the presence of FGF2 as adherent monolayers. We hypothesized that an alteration of the rates of NPC proliferation could result in early brain overgrowth. Proliferation was measured by calculating the population doubling time from plating at passages 3 to 7 (P3-7) in continuous culture. From P4, the population doubling time decreased in ASD NPCs from all 8 patients compared to NPCs from all 5 controls, reaching statistical significance at P6 (**Figure 3a**). Cell cycle analysis at P6 revealed that shortening of G1 and S phases was the main reason for the decrease in the

population doubling time; with no change of G2-M phase length (**Figure 3b**). Double labeling for Ki67 and pHH3 revealed an increased percentage of $Ki67^+$ (cycling cells) in ASD relative to control NPCs, whereas the percentage of $pHH3^+Ki67^+$ (G2-M phase mitotic cells) was unaffected in autistic NPCs (**Figure 3c, d**). These findings demonstrate that iPSC-derived NPCs from ASD patients with macrencephaly proliferated faster than those derived from controls. Next, we characterized the expression of forebrain and midbrain markers in the ASD-derived NPCs and those from control individuals. SOX1 (a marker for maintenance of radial glial NPCs identity), and Vimentin (the intermediate filament protein of NPCs) were up-regulated in the ASD NPCs compared to control NPCs (**Figure 3e-i)**. SOX1 and Vimentin were previously shown to correlate with induced proliferation of NPCs [3,4], consistent with the increased rate of proliferation of NPCs derived from the ASD individuals. The expression of PAX6 (a marker of forebrain neural-ectoderm) and OTX2 (a marker of the midbrain and forebrain), measured by immunocytochemistry, Western blot and qPCR, were unchanged in ASD and control NPCs (**Figure 1g-k and Supplementary Figure 2**). In addition, the expression of the intermediate NPCs marker TBR2 was low and did not differ between ASD and control NPCs (data not shown). We then investigated further the mechanism generating increased progenitor proliferation in ASD cohort. Based on previous unpublished observations we hypothesized that the aberrant proliferation in ASD-derived NPCs could be mediated by a deregulation of the b-catenin/Brn2/Tbr2 transcriptional cascade. To test our hypothesis, we first examined Wnt/b-catenin transcriptional activity using TOP-flash assays. β-catenin transcriptional activity was reduced in untreated ASD compared to control NPCs (**Figure 2a**). Activation of canonical Wnt signaling with 5 mM LiCl, which prevents GSK3-mediated degradation of b-catenin, elevated b-catenin transcriptional activity in ASD and control NPCs. However, b-catenin transcriptional activity was significantly reduced in LiCl-treated ASD NPCs compared to control NPCs (**Figure 2a**), indicating that the cause of reduced b-catenin transcriptional activity is downstream of GSK3. Activation of canonical Wnt signaling with Wnt3A resulted in similar, marked but non-significant elevation of b-catenin transcriptional activity in both control and ASD NPCs (**Figure 2a**), although a similar trend of reduced b-catenin transcriptional activity in the ASD NPCs was noted. These findings indicate that the b-catenin transcriptional activity was reduced in ASD-derived NPCs.

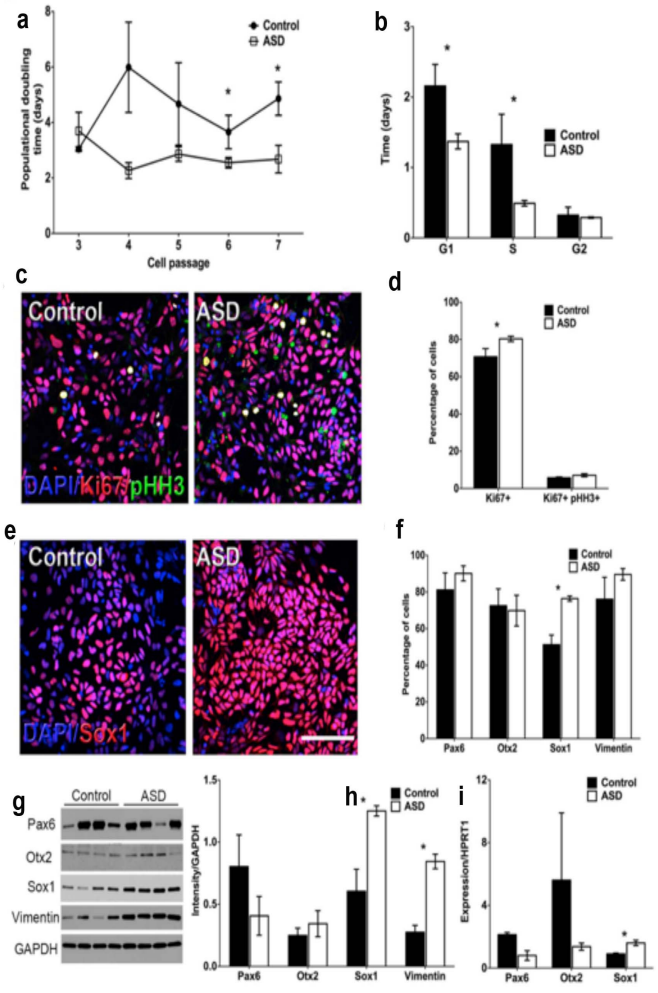

Figure 3: (**a**) iPSCs from ASD and control were differentiated to NPCs. From passages 2 to 6 cells were plated at the same density and population-doubling time at each passage was calculated. Results of 5-6 lines (2 clones per line) are presented as mean±SEM (*p=0.02). (**b**) Adherent monolayer NPCs from control and ASD iPSCs were dissociated, counted for calculation of population doubling time and prepared for cell cycle analysis. Results are presented as the time spent in each cell cycle stage (n≥4, mean±SEM, ANOVA p<0.04, post-hoc p<0.04 for comparing the time spent in G1 phase in the ASD NPCs with those of the control NPCs, respectively). (**c**) Control and ASD NPCs were immunostained with DAPI (Blue), anti-pHH3 (Green) and anti-ki67 (Red) (Scale bar: 200um), Representative images of the staining are shown. (**d**) Quantification of the percentage of $Ki67^+$ and $Ki67^+pHH3^+$ labeled cells are presented as mean±SEM (n≥5; *p<0.03 for comparing the results of the ASD with those of the control NPCs). (**e**) Representative images of the NPC staining for Sox1 (Scale bar: 200mm). (**f**) Quantification of the percentage of $Pax6^+$, $OTX2^+$, $Sox1^+$ and $Vimentin^+$ labeled cells is presented as mean±SEM (n≥5; *p<0.006 for comparing the results of the ASD with those of the control NPCs). (**g**) Representative immunoblot of control and ASD-derived NPCs. (**h**) The levels of Pax6, OTX2, Sox1 and Vimentin, which were normalized to GAPDH levels, were quantified and results are presented as mean±SEM (n≥5; *p<0.007 for comparing the results of the ASD with those of the control NPCs). (**i**) RNA of control and autistic NPCs was extracted and RT-PCR was performed. The levels of Pax6, OTX2, Sox1, which were normalized to HPRT1, are presented as mean±SEM (n≥3; *p<0.04 for comparing the results of the ASD with those of the control NPCs).

The NPC lines from all patients and controls were generated at Dr. Gage laboratory, Salk Institute, La Jolla, CA; all the proliferation tests and β-catenin/BRN2 transcriptional activity tests, immunocytochemistry, western blot and qPCR experiments described on Task1b were performed in the lab of Dr. Wynshaw-Boris laboratory, UCSF, San Francisco, CA / Case Western Reserve University, Cleveland, OH.

1c. *Perform neuronal differentiation using the iPSC clones characterized in 1a and analyze neuronal maturation using the methods described below (months 8-16).*

We performed differentiation experiments to begin analyzing the neuronal maturation in ASD versus controls. We hypothesized that the proliferation defects observed in ASD NPCs could result in abnormal neuronal differentiation. Therefore, we examined the earliest stages of neuronal differentiation in control and ASD NPCs following 1, 2 and 4 weeks of differentiation. Differentiated NPCs were fixed at the indicated time points and immunocytochemistry was performed for TUJ1 and Doublecortin (DCX) (**Figure 4a**). After 1 week of differentiation, the percentages of TUJ1 and DCX were higher in the ASD samples compared to controls (**Figure 4b, c**). The levels of TUJ1 protein after one week of differentiation were also elevated in ASD compared to control samples by Western blot analysis (**Figure 4d, e**), similar to immunocytochemistry, supporting premature differentiation of ASD NPC. However, differentiation for 2 or more weeks did not reveal differences in the percentage of differentiated cells between ASD and control samples (**Figure 4a-c**). Evidence from the literature suggests an imbalance in excitatory versus inhibitory signals in developing ASD [5,6]. To examine whether the ASD-NPCs led to a change in excitatory versus inhibitory cell fate, we measured Glutamatergic (NGN2) and GABAergic (MASH1, DLX2, OLIG2 and NKX2.1) progenitor markers in ASD and control NPCs. A reduction in the percentage of $NGN2^+$ NPCs in ASD NPCs compared to controls was observed (**Figure 4f, g**). In contrast, markers of inhibitory precursors present in the subpallium (MASH1, DLX2 and NKX2.1) were up-regulated in ASD compared to control NPCs. However, OLIG2, another subpallium marker, was unchanged between control and ASD NPCs (**Figure 4g**). Together the results described above indicate that ASD neurons start the differentiation program earlier and that could have important implications to proper neuronal maturation and function. We are now testing the synaptogenesis and neuronal function (i.e. electrophysiology) from mature neurons (6-8 weeks after differentiation) and will present the results on the next progress report.

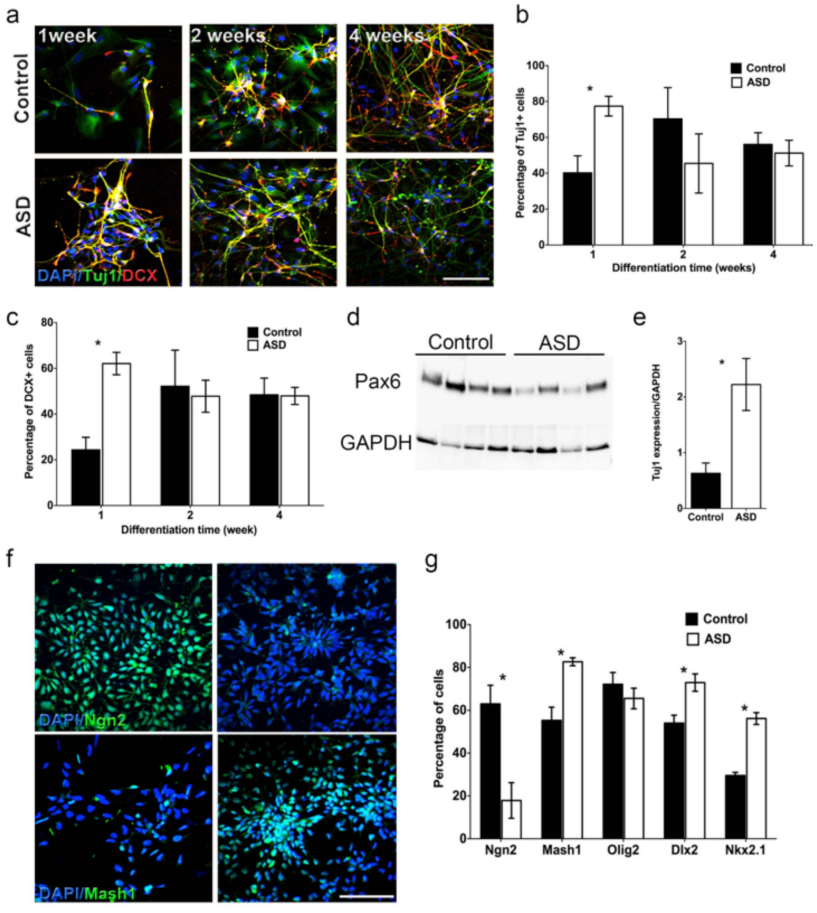

Figure 4. Neuronal differentiation of iPSCs. (**a**) Control and ASD NPCs were differentiated into neurons and fixed after 1, 2 and 4 weeks. (Scale bar: 200mm). The percentages of $Tuj1^+$ (**b**) and DCX^+ (**c**) cells were measured and results are presented as mean±SEM (n=4, AVOVA<0.02 *p<0.04, comparing the results of ASD neuron with those of the control and neurons). (**d**) Control and ASD NPCs were differentiated for 1 week after which cells were lysed and immunoblot for Tuj1 and GAPDH and representative blot is presented. (**e**) The levels of Tuj1, which were normalized to GAPDH levels, were quantified and results are presented as mean±SEM (n=4; *p<0.02 for comparing the results of the ASD with those of the control neurons). (**f**) Representative images of the control and ASD NPCs immunostained for Ngn2 and Mash1. (Scale bar: 200 mm). (**g**) Quantification of the percentage of $Ngn2^+$, $Mash1^+$, Olig2, $Dlx2^+$ and $Nkx2.1^+$ labeled cells is presented as mean±SEM (n≥5; *p<0.03 for comparing the results of the ASD with those of the control NPCs).

All the neuronal differentiation assays, immunocytochemistry, western blot and qPCR experiments described on Task1c so far were performed in the lab of Dr. Wynshaw-Boris laboratory, UCSF, San Francisco, CA / Case Western Reserve University, Cleveland, OH.

Progress report on **Task 2: Functional characterization of dysregulated pathways uncovered by gene expression from iPSC-derived NPCs and neurons (months 16-24)** will be presented in the next period.

4. **KEY RESEARCH ACCOMPLISHMENTS:**

We are excited to report 2 key great research accomplishments performed in the first period of the grant, highlighted bellow:

1- We demonstrated that iPSC-derived NPCs from ASD patients with macrencephaly proliferated faster than those derived from controls and that can be explained by a reduction in β-catenin transcriptional activity.

2- We showed that ASD neurons start the differentiation program earlier and that could have important implications to proper neuronal function maturation to be further investigated.

5. **CONCLUSION:**

The use of iPSCs to study genetic disorders is a powerful tool to dissect molecular and cellular pathways implicated in disease pathology during early stages of human neurodevelopment. However, modeling highly complex idiopathic disorders such as ASD is challenging due to a high level of heterogeneity in the patient population. Here, we took advantage of iPSCs derived from a carefully characterized clinical cohort of ASD patients who have an anatomical phenotypic trait that occurs in about 20-30% of idiopathic ASD: an early developmental enlargement of brain volume, including macrencephaly that is frequently associated with poor prognosis. We reasoned that ASD patients sharing a common phenotype, early developmental brain enlargement ranging from mild to extreme macrencephaly, might share underlying molecular and cellular pathway dysregulation. Our initial findings described in the first round of this grant show that neural progenitor cells (NPCs) derived from ASD-iPSC proliferated faster than those derived from controls and showed reduction in β-catenin transcriptional activity. In addition, we showed that ASD neurons start the differentiation program earlier and that could have important implications to proper neuronal maturation and function.

In the next year we will focus on testing the synaptogenesis and neuronal function of mature neurons (i.e. percentage of excitatory and inhibitory neurons, expression of synaptic markers and electrophysiology) and on transcription profiling (RNA expression assessment) of ASD NPCs and neurons versus control cells. Together, our initial results suggest that idiopathic ASD can be modeled using the iPSC technology to reveal novel cellular and molecular mechanisms underlying brain abnormalities.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**

MANUSCRIPT IN PREPARATION

Marchetto, M.C., Belinson, H., Tian, Y., Beltrao-Braga, P., Trujillo, C., Mendes, A., Nunez, Y., Gosh, H., Brennand, K., Pierce, K., Pramparo, T., Eyler, L., Barnes, C.C., Courchesne, E., **Gage, F.H.**, Geschwind, D., Wynshaw-Boris, A.* and Muotri, A.R.* Evidence for proliferation and synaptogenesis impairments in neural cells derived from idiopathic autistic patients. * co-corresponding authors

7. **INVENTIONS, PATENTS AND LICENSES:** Nothing to report

8. **REPORTABLE OUTCOMES:** Nothing to report

9. **OTHER ACHIEVEMENTS:**

This award contributed to the production of control and ASD iPS cell lines from 13 individuals listed bellow (and 2 clones each, except for CLUE).

1-AQUA, 2-AHOY, 3-ACAI, 4-AVID, 5-ABLE, 6-AERO, 7-ARCH, 8-APEX (ASD)

9-CHAP, 10-CLAY, 11-COVE, 12-CENT, 13-CLUE (Control)

10. **REFERENCES:**

Takahashi, K. *et al*. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861-872 (2007).
Marchetto, M. C. *et al*. A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. *Cell* 143, 527-539, doi:S0092-8674(10)01186-4 [pii] 10.1016/j.cell.2010.10.016 (2010).
Elkouris, M. *et al*. Sox1 maintains the undifferentiated state of cortical neural progenitor cells via the suppression of Prox1-mediated cell cycle exit and neurogenesis. *Stem Cells* 29, 89-98, doi:10.1002/stem.554 (2011).
Alonso, G. Proliferation of progenitor cells in the adult rat brain correlates with the presence of vimentin-expressing astrocytes. *Glia* 34, 253-266 (2001).
Rubenstein, J. L. Three hypotheses for developmental defects that may underlie some forms of autism spectrum disorder. *Curr Opin Neurol* 23, 118-123, doi:10.1097/WCO.0b013e328336eb13 (2010).
Zikopoulos, B. & Barbas, H. Altered neural connectivity in excitatory and inhibitory cortical circuits in autism. *Frontiers in human neuroscience* 7, 609, doi:10.3389/fnhum.2013.00609 (2013).

11. **APPENDICES:** Nothing to report